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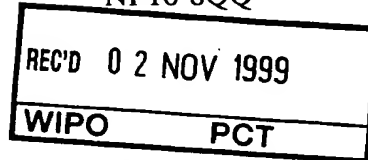
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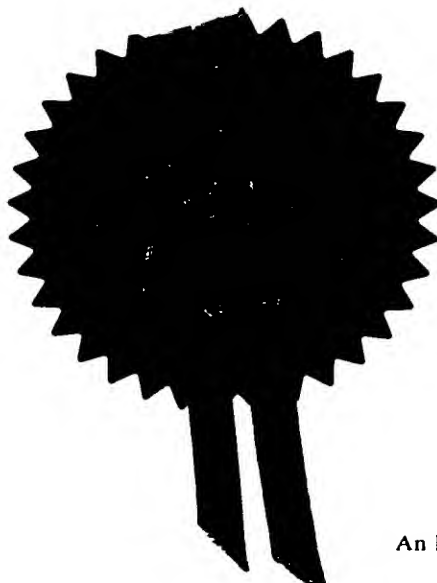


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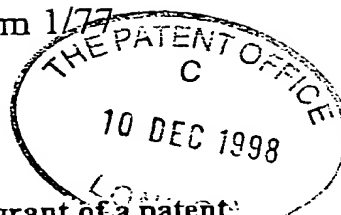


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Request for grant of a patent

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2.	Patent application number (The Patent Office will fill in this part)	10 DEC 1998	9827243.8	
3.	Full name, address and postcode of the or of each applicant ( <i>underline all surnames</i> )	The University of Bath Claverton Down BATH BA2 7AY		
	Patents ADP number ( <i>if you know it</i> )	798157001		
	If the applicant is a corporate body, give the country/state of its incorporation	United Kingdom		
4.	Title of the invention	Compositions comprising xanthine oxidoreductase		
5.	Name of your agent ( <i>if you have one</i> )	Abel & Imray		
	"Address for service" in the United Kingdom to which all correspondence should be sent ( <i>including the postcode</i> )	20 Red Lion Street LONDON WC1R 4PQ		
	Patents ADP number ( <i>if you know it</i> )	174001		
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8.	Is a statement of inventorship and of right to grant of a patent required in support of this request? ( <i>Answer 'Yes' if:</i> <i>a) any applicant named in part 3 is not an inventor, or</i> <i>b) there is an inventor who is not named as an applicant, or</i> <i>c) any named applicant is a corporate body.</i>	Yes		

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Description 26

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Abstract

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Statement of inventorship and right to grant of a patent (*Patents Form 7/77*)

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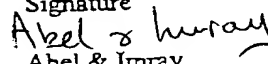
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Date

10 December 1998

12. Name and daytime telephone number of person to contact in the United Kingdom

Dr. Ceris Humphreys

01225 469914

THE UNIVERSITY OF BATH

CASE NO. 4464 GB .

"COMPOSITIONS COMPRISING XANTHINE OXIDOREDUCTASE"

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LONDON, WC1R 4PQ.

Compositions comprising xanthine oxidoreductase

The present invention relates to compositions  
5 comprising xanthine oxidoreductase (XOR). In particular,  
but not exclusively, the invention relates to the use of  
a composition comprising XOR as a feed for humans or  
animals, and finds special application in relation to  
formula feed for babies.

10 The enzyme xanthine oxidoreductase (XOR) is a  
complex molybdoflavoprotein, the action of which has been  
studied for many years.

XOR is a major protein component of the membrane  
surrounding fat droplets in whole milk. Consequently,  
15 cows' milk is a rich and convenient source of the enzyme,  
as has been known for many years. XOR has also been  
characterised from rat, chicken and turkey livers. More  
recently, human milk has been found to contain XOR, which  
has now been purified from that source. Initial research  
20 shows that, while the human milk XOR enzyme has similar  
physicochemical properties to the bovine milk enzyme, the  
human enzyme shows differences in its catalytic activity.

The purpose of XOR in milk has never been fully  
ascertained. One suggestion has been that it provides a  
25 source of molybdenum and iron, metals potentially useful  
for the developing neonate.

There have also been suggestions that XOR has a role  
in the production of bactericidal agents. As discussed  
further below, XOR can catalyse the production of  
30 superoxide and hydrogen peroxide, which are known  
bactericidal agents. As indicated below, however, in the  
low oxygen concentrations found in the gut, it is thought

unlikely that bactericidal levels of superoxide or hydrogen peroxide will be attained.

The role of XOR in the development of gout has been the subject of extensive study. XOR is involved in the catabolism of purines to uric acid, catalysing the oxidation of hypoxanthine to xanthine and xanthine to uric acid. Compositions have been developed which block the action of XOR and which are useful in the prevention and treatment of gout.

XOR exists in two inter-convertible forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XO). XDH, which is believed to predominate *in vivo*, preferentially reduces  $\text{NAD}^+$ , whereas XO does not reduce  $\text{NAD}^+$ , preferring molecular oxygen. Where reference is made herein to xanthine oxidoreductase (XOR - E.C. 1.2.3.2), that term includes xanthine oxidoreductase, which is to be understood as referring to both xanthine dehydrogenase (XDH) and xanthine oxidase (XO), where appropriate. It will be appreciated that references to XOR further include references to analogs of xanthine oxidoreductase that have xanthine oxidoreductase activity. Such analogs may include but are not limited to, for example, xanthine oxidoreductase which has been modified chemically or otherwise, analogs having fragments of xanthine oxidoreductase derived from naturally-occurring enzyme, and analogs having polypeptides obtained by replication of the enzyme or a portion thereof using any suitable biotechnological method, provided in each case that the catalytic activity of the endogenous xanthine oxidoreductase is retained at least to an appreciable extent.

XOR can also effect reduction of molecular oxygen. That reaction generates the reactive oxygen species superoxide anion and hydrogen peroxide.

The superoxide radicals thus generated have been  
5 implicated in relation to chronic inflammatory intestinal diseases such as Crohn's disease and colitis ulcerosa. US 5,484,605 describes the administration of oxypurinol to the intestine to inhibit xanthine oxidase, thus preventing the formation of superoxide radicals which are  
10 believed to act as inflammatory mediators.

It has been reported, Millar, T.M. et al, FEBS Letters 427 (1998) 225-228, that, under hypoxic conditions and in the presence of NADH, XOR is capable of catalysing the reduction of glyceryl trinitrate (GTN), as  
15 well as inorganic nitrate and nitrite, to nitric oxide (NO).

Nitric oxide (NO) is widely recognised as mediating the relaxation of smooth muscle in vasodilation and as initiating many other important biological functions,  
20 including inhibition of platelet aggregation and adhesion. Its generally accepted physiological source is NO synthase, a complex enzyme which is totally dependent on oxygen for its activity and consequently ineffective in a hypoxic environment, where the vasodilatory  
25 properties of NO might be seen to be advantageous.

Babies who are not breast-fed are fed what is referred to herein as formula feed. Such formula feed has a composition which commonly includes sources of protein, fat and carbohydrate as well as minerals and are  
30 generally formulated to be nutritionally complete. Many formula feeds are based on cow's milk while some others are soy based. The term "formula feed" used herein should be understood to cover both formulations based on



milk products as well as those based on soya or other products and which may or may not be nutritionally complete. The formula feed commonly takes the form of a dried powder which is reconstituted before being fed to the neonate. Alternatively, the formula feed may be in liquid form either as a concentrated liquid requiring dilution or as a ready-to-feed formulation.

Enteral feeding may be prescribed where a patient is unable to eat normally, or has severe malabsorption or malnourishment. Enteral feed may take the form of tube and sip feeds. The enteral feed may be nutritionally complete and generally comprises protein, carbohydrate and fat as well as vitamins and minerals. The term "enteral feed" used herein should be understood to cover both formulations based on milk products as well as those based on soya or other products and which may or may not be nutritionally complete. The enteral feed may be in liquid form or may be in the form of a powder which is reconstituted before use. In many cases the enteral feed formulation is based on the composition of the neonatal formula feed.

The term "feed" is used herein to include both enteral feeds and formula feeds.

In accordance with the invention, there is provided, a formulation for use as a feed, the formulation including active xanthine oxidoreductase (XOR).

We have found that the active enzyme xanthine oxidoreductase (XOR) is absent from formula feeds and from enteral feeds. It is thought that that is because XOR is either absent from the feed or it is inactivated as a result of the treatment process used in the production of feeds. Where reference is made herein to "active" XOR and "active" enzyme, it is to be understood

that reference is made to XOR and enzyme which has not, for example, been inactivated or broken down in such a way.

Up to now, it would have been thought that the addition of active XOR to feeds would be unnecessary and undesirable. The only roles of XOR previously known, as indicated above, were as a general "housekeeping" enzyme in the catabolism of purines, which may also have a pathological role in the development of gout and of chronic inflammatory intestinal diseases. The only other beneficial role of XOR was thought to be as a source of molybdenum and iron. It was thought (correctly) that those minerals could still be obtained from inactivated XOR, which might be present in feed.

We have now found that the addition of active XOR to feed is potentially beneficial in killing pathogenic intestinal bacteria. It is believed that feed containing active XOR will be beneficial in the mitigation of intestinal infection and necrotising enterocolitis in the neonate fed with formula feed. Sick adults that are enterally fed are also at risk of the same complications as children fed formula feed.

Studies have shown that babies who are fed with formula feed are about twenty times more likely to suffer from gastrointestinal (GI) infection than babies who are breast fed. The reason for that discrepancy has not been known.

In the presence of oxygen, as stated above, XOR can generate superoxide and hydrogen peroxide. However, the amount of oxygen available in the gut is generally very low and bactericidal levels of superoxides or hydrogen peroxide are unlikely to be attained.

As discussed above, it has been found that the catalysis by XOR of the reduction of glyceryl trinitrate (GTN), as well as inorganic nitrate and nitrite, to nitric oxide (NO) occurs under hypoxic conditions. It is  
5 such hypoxic conditions which are present in the gut.

We have also found that the optimum pH for the production of NO under the hypoxic conditions is about pH 5.5. It is known that the pH of the neonatal stomach normally ranges between pH 3.5 and 6. Thus, we have  
10 found that the neonatal stomach presents an environment in which the production of NO in the presence of XOR is at a peak. Furthermore, it is at pH levels above about 4 at which pathogenic bacteria are more active than at lower pH of about 2 normally found in the adult gut.  
15 Thus it is believed that the neonatal stomach, having a relatively high pH, is at particular risk from pathogenic bacteria. Furthermore, it is found that, for example in post-operative adults, the pH of the stomach rises to above 4. It has also been noted that post-operative  
20 adults have a relatively high risk of GI infection.

Thus, surprisingly, we have found under the conditions of the neonatal gut, XOR can catalyse the production not only of superoxide, but of NO. Superoxide and NO rapidly interact to generate peroxynitrite, a much  
25 more potent bactericidal species than superoxide, NO or hydrogen peroxide. While superoxide has some bactericidal properties and in some situations NO has also been found to kill or damage bacteria, it is the interaction of superoxide and NO to form peroxynitrite  
30 and other products which is believed to give superior bactericidal action. Peroxynitrite (and, it is thought, other products of the interaction of superoxide and NO) are particularly potent bactericidal species.

In one embodiment of the invention, the formulation is for use as formula feed. As indicated above, the use of XOR finds particular application in relation to the feeding of neonates.

5       Alternatively, the formulation is for use as an enteral feed for adults.

While reference is made herein to the feeding of humans, the invention is also of particular relevance in the feeding of animals and the terms "formula feed",  
10   "enteral feed" and "feed" should be understood to include formulations for animals. The invention finds particular application in respect of mammals.

Animals also suffer from GI infection, in particular Scours disease (diarrhoea in neonatal animals). Scours  
15   disease is a particular problem for calves and pigs taken from their mothers soon after birth. For calves and pigs taken from their mothers up to ten days after birth and fed waste milk or formula feed, the mortality rate can be as much as 80%. In some cases, Scours disease can be  
20   cured by administering electrolyte solutions but such treatment is very expensive.

It is thought that the addition of active XOR to feed for animals will mitigate GI infections, in particular Scours disease.

25       Advantageously, the formulation includes from 50 to 500 $\mu$ g/ml of XOR, based on the volume of the formulation (when ready-to-use, having been diluted if necessary). Preferably the formulation includes from 50 to 150 $\mu$ g/ml which is comparable to the level of XOR found in natural  
30   breast milk. In some cases, it may be desirable to increase or reduce the amount of active XOR in the feed, for example, as a function of the baby's age and/or the incidence of GI infection.

We have found that a particularly advantageous source of active XOR is buttermilk, in particular lyophilised buttermilk.

5 In some cases it is thought that the addition of XOR in purified or other form will be desirable, for example where there is a risk of allergy to buttermilk.

The formula feed and/or the enteral feed including the active XOR in accordance with the invention may be in the form of a powder or may be in liquid form ready for  
10 feeding to the patient.

As indicated above, it is believed that for the formula feed and enteral feed formulations, any active XOR which was naturally present in those formulations is destroyed or deactivated during the manufacture of the  
15 formulation, possibly as a result of heat treatment. In a particularly preferred embodiment of the invention, one portion of the formula or enteral feed is prepared in the standard way, for example including heat treatment step(s). A composition comprising active XOR is  
20 subsequently added to the prepared portion. That addition of the XOR may be carried out by a manufacturer, or may be carried out, for example, immediately prior to use of the formulation. For example, in the case of a powdered feed which is made up with water prior to use, a  
25 powdered composition comprising the active XOR may be contained in a separate container from that of the rest of the formulation, for example in a sachet. Alternatively, the portion including the active XOR may be in tablet form or may be contained in a capsule. The  
30 rest of the feed may be made up in the normal way, for example by the addition of hot water and shaking, and the XOR composition added once the rest of the feed has been prepared. That is of particular importance in the case

in which the normal method of making up of the formulation might deactivate the XOR, for example as a result of the addition of boiling water. Where the making up of the formulation would not damage the activity of the XOR, or where the formulation is sold ready-prepared, the XOR composition and the rest of the formulation may be provided together as a mixture.

Thus the invention provides a formulation which comprises two separate portions, the first portion including active XOR and the second portion comprising substantially no active XOR.

In one embodiment of the invention, the second portion of the formulation is in the form of a powder.

As indicated above, the second portion may have been treated by heat treatment, for example UHT treatment, in the normal way, thus deactivating the XOR.

In a particularly preferred embodiment of the invention, the active XOR is added in the form of buttermilk. Advantageously, the first portion has been pasteurised. As indicated above, pasteurisation has been found not to deactivate XOR.

As indicated above, advantageously, the first portion is in a first container, the second portion is in a second container. Thus the two portions can be held separate until use. For example, where both portions are in the form of a powder, each powder portion can be made up separately using different methods before being mixed together. For example, the first portion might be made up with cold water and the second portion may be made up with boiling water. Alternatively, the first portion may be in the form of a liquid ready for mixing into the second portion of the formulation once the second portion has been made up.

Advantageously, the formulation further includes electron donors and/or electron acceptors. Examples of electron donors are purines and nitrogen-containing heterocyclic compounds, for example NADH. Examples of  
5 electron acceptors are organic and inorganic nitrates and nitrites.

According to the invention, there is also provided, a composition for addition to a formulation for use as feed, the composition comprising active XOR. It is  
10 envisaged that the composition containing the active XOR might be sold separately from the formula or enteral feed, the composition being for addition to the feed prior to use.

Advantageously, the composition comprises  
15 buttermilk. As indicated above, buttermilk is a particularly preferred source of active XOR. Preferably, lyophilised buttermilk is used.

The composition may be in the form of a powder.

Advantageously, the composition further includes  
20 electron donors and/or electron acceptors. Examples of electron donors are purines and nitrogen-containing heterocyclic compounds, for example NADH. Examples of electron acceptors are organic and inorganic nitrates and nitrites.

25 According to the invention, there is also provided, a method of making a formulation for use as feed, the method comprising the step of adding a composition comprising active XOR.

Advantageously, the composition is as described  
30 above.

The method advantageously comprises the steps of:

a. preparing a first portion of the formulation,  
the first portion comprising a composition including  
active XOR; and

b. preparing a second portion of the formulation,  
5 the first portion and second portion being separate from  
each other for subsequent mixing to form the formulation.

Advantageously, the first portion comprises  
buttermilk, preferably lyophilised buttermilk. As  
indicated above, the buttermilk may include additives.

10 Preferably, the second portion comprises a  
sterilised feed composition.

Also provided by the invention is the use of active  
XOR in the treatment of gastrointestinal (GI) infection.  
The invention also provides the use of active XOR in the  
15 treatment of Scours disease.

Additionally provided is the use of active XOR in  
the killing of bacteria.

The invention also provides a method of feeding a  
patient with enteral feed, in which the enteral feed  
20 includes active XOR and also a method of feeding a  
neonate with formula feed, in which the formula feed  
includes active XOR.

Also provided is a method of treatment of  
gastrointestinal infection using active XOR.

25 It will be appreciated that XOR used in accordance  
with the invention may be of any biological origin, for  
example of mammalian or other animal origin, or  
originating from a suitable micro-organism, for example,  
aspergillus sp. XOR of ruminant origin offers the  
30 advantage of ready availability.



Embodiments of the invention will now be described, by way of example, with reference to the accompanying drawings, of which:

Fig. 1 is a graph illustrating the effect of peroxynitrite on cell viability, under the conditions of Test 8(a);

Fig. 2 is a graph illustrating the effect of added peroxynitrite on cell colony growth in semi-skimmed-bovine milk, as determined in Test 8(b); and

Fig. 3 is a graph illustrating the dependence of XO-mediated killing of cells as determined in Test 8(c).

#### Determination of nitric oxide production

The production of nitric oxide in the following tests and examples was analysed using an ozone chemiluminescence assay in a continuous flow apparatus (Sievers NOA 280) that allows the real time analysis of NO production. The apparatus was modified to allow a constant stream of nitrogen to flow into the reaction chamber.

Chemiluminescence data were collected by a data acquisition system; the mean NO produced in parts per billion (ppb) was calculated from readings taken every second and shown as NO ppb/s.

Progress curves, of molar production of NO against time, were calculated by taking into account the gas flow and successively integrating the ppb/s curves. Reactions were carried out in a final volume of 1 ml at 37°C in an atmosphere of < 1% oxygen (Stathkelvin combination needle oxygen electrode, Diamond General Corp.).

The method used was as follows:

(a) Two clean 7ml bijous were obtained, one for each of the "substrates" and the "milk".

To the "substrates" bijou, 200 $\mu$ l of 100mM stock sodium nitrite was added together with 200 $\mu$ l of the 5mM reduced NADH to give an assay concentration of 20mM nitrite and 1mM reduced NADH.

- 5 To the "milk" bijou were added 600 $\mu$ l of milk sample to give an assay volume of 1ml.

(b) Using a flow rate of 200ml/min, each bijou mixture and a corresponding injection needle was degassed with nitrogen gas ( $N_2$ ) for about 10 seconds and the bijou  
10 was capped.

(c) The reaction cell comprised a 7ml screw-cap bijou having three needle holes in its cap. A continuous flow of warmed  $N_2$  (200ml/min flow rate) was injected into the bijou through one of the needle holes in the cap to  
15 give the required hypoxic conditions. The reaction cell was held at a temperature of 37°C in a water bath mounted on a magnetic stirrer. A magnetic flea was placed in the reaction cell to mix the samples once injected.

(d) Samples of NO were taken from the reaction cell  
20 by a needle that was connected to a Sievers Nitric Oxide Analyser (NOA-280) and the results were recorded for analysis using a computer.

(e) At time (t) 0 minutes, the NOA-280 started the measurement of NO from the reaction cell. At t=1 minute,  
25 the contents of the "substrates" bijou were injected into the reaction cell using a 1ml syringe and the background NO was measured.

(f) At t=5 minutes, the contents of the "milk" bijou were injected into the reaction cell using a 1ml syringe  
30 and the release of NO was monitored for a further 20 minutes. The set up was such that the reaction cell was a sealed system having an inlet gas flow of 200ml/min and a sample extraction flow to the NOA-280 of 200ml/min.

The steady-state generation of NO (which corresponded to a plateau region on the trace of the NO production) was noted to give the mV/s release of NO from the 1ml assay volume.

- 5        The samples were diluted with PBS where necessary to give an assay of 1ml for the test of NO generation.

#### Reagents used

- 10        The reagents used in the tests and examples described below were as follows:

1. Bovine xanthine oxidase (XOR) - Biozyme, Blaenavon, UK.  
This source of enzyme had a concentration of 10.7mg/ml and was batch 104AX
- 15    2. Sodium nitrite ( $\text{NaNO}_2$ ) - Sigma Chemicals (Sigma-Aldrich Company Ltd.), Poole, UK. This was dissolved in 1X Phosphate Buffered Saline (PBS), pH 7.3, to the required concentration.
3.  $\beta$ -Nicotinamide Adenine Dinucleotide, reduced form, ( $\beta$ -  
20    NADH) - Sigma Chemicals.
4. Phosphate Buffered Saline (PBS) - Oxoid Ltd., Basingstoke, UK. Tablets were added in the proportion of 1 per 100ml of distilled water and mixed until thoroughly dissolved to give 1X PBS, pH7.3.
- 25    5. Oxypurinol - Sigma Chemicals. A stock 1mM solution was made up by adding 0.0015g to 0.25ml of 1M NaOH. This was mixed until the oxypurinol dissolved. Then 9.75ml of 1X PBS was added and the pH altered until pH7.3 was reached using drops of 1M HCl.
- 30    6. Diphenyliodonium (DPI) - ICN Biomedical. A stock 1mM solution was made up by adding 0.0032g to 10ml 1X PBS, pH7.3.
7. Formula milks:

- a. PreAptimil with Milupan (2.4g protein/100ml) - Milupa, Trowbridge, UK.
- b. Aptamil First with Milupan (1.5g protein/100ml) - Milupa.
- 5 c. Farleys First Milk - H.J. Heinz Co. Ltd., Hayes, UK.
- d. Cow and Gate Premium (1.4g protein/100ml) - Cow and Gate, Trowbridge, UK.
- e. Sma Gold 1.5g protein/100ml) - SMA Nutrition, Maidenhead, UK.
- 10 f. Sma Wysoy (1.8g protein/100ml) - SMA Nutrition.
8. Human Breast Milk. Samples obtained from subjects in the local area
9. Carton milk. Milk bought in pints from the local
- 15 shop either as full milk or semi-skimmed milk (3.4g protein/100ml). Lordswood Dairy, Bristol, UK was the source of the milk.
10. Untreated cows' milk was obtained from a local farm
11. Buttermilk obtained from Waitrose (John Lewis
- 20 Partnership, UK).

#### Test 1

The release of nitric oxide (NO) from a composition including pure bovine xanthine oxidase under hypoxic conditions was studied. The samples studied comprised reduced  $\beta$ -Nicotinamide adenine dinucleotide (NADH) and nitrite ( $\text{NO}_2^-$ ) and pure bovine xanthine oxidase.

Bovine xanthine oxidase was diluted with PBS as indicated in Table 1 below to give 300 $\mu$ l of enzyme mixture. The enzyme mixture (300 $\mu$ l) was mixed with 700 $\mu$ l of a substrate mixture to give an assay volume of 1 ml. The substrate mixture comprised nitrite which was added

to the assay to give a concentration of 1mM and NADH which was added to give a concentration of 0.3mM. Where, for example in Tests 2 to 4 below, additional components are added to the sample, the amount of PBS is adjusted accordingly to give an assay volume of 1 ml. The total XOR protein in the assay is shown in Table 1. The sample was placed in the reaction vessel of the NO determination apparatus under a nitrogen atmosphere and the generation of NO was measured and the results calculated as a steady state rate in mV/s. The rate of NO release for the different concentrations of XOR is also shown in Table 1.

Table 1

Volume XOR enzyme ( $\mu$ l)	Volume PBS ( $\mu$ l)	XOR protein in assay ( $\mu$ g)	NO release (mV/s)
0	300	0.0	15.10
2	298	21.4	50.05
5	295	53.5	76.35
10	290	107.0	128.55
15	285	160.5	187.20
20	280	214.0	225.75

15

Where the pure XOR is used in the samples, NADH was required as a substrate for the reduction of the nitrite to proceed. It is believed that in many cases, where the XOR is added to, for example a formula feed, the addition of a separate electron donor, for example NADH, will not be required because suitable substrate species will already be present in the feed and/or in the GI tract of the infant or adult to whom the feed is administered.

25

### Test 2

Using the method described above in respect of Test 1, the effect of the variation in the concentration of NADH on the production of NO was investigated. 4mM NADH was  
5 diluted with PBS to give the relevant concentration. The results are shown in Table 2.

Table 2

XOR protein in assay ( $\mu$ g)	NADH concentration in assay (mM)	NO release (mV/s)
53.5	0.00	0.00
53.5	0.10	30.45
53.5	0.25	66.80
53.5	0.50	92.55
53.5	1.00	103.35
53.5	2.00	109.20
107.0	0.00	0.00
107.0	0.10	74.45
107.0	0.25	142.70
107.0	0.50	192.65
107.0	1.00	230.05
107.0	2.00	264.65

10

### Test 3

Using the method described above in respect of Test 1, the effect of the variation in the concentration of  
15 nitrite on the production of NO was investigated. As for Test 1, the concentration of NADH was 0.3 mM. 1M nitrite (or 100mM nitrite, where appropriate for low

concentrations) was diluted with PBS to give the relevant concentration. The results are shown in Table 3.

Table 3

5

XOR protein in assay ( $\mu\text{g}$ )	Nitrite concentration in assay ( $\text{mM}$ )	NO release ( $\text{mV/s}$ )
21.4	0	0.00
21.4	1	53.75
21.4	5	184.8
21.4	10	378.10
21.4	25	407.55
21.4	50	474.05

Test 4

- 10 The method of Test 1 was repeated with known inhibitors of XOR included in the assay to show that the NO generation was being catalysed by XOR. Two different inhibitors were used. Oxypurinol was used at a concentration of 100  $\mu\text{M}$ . Oxypurinol is a molybdenum  
15 site-specific inhibitor. Diphenyliodonium (DPI) was used at a concentration of 100  $\mu\text{M}$ . DPI is a FAD site inhibitor. The results are shown in Table 4.

Table 4

Inhibitor	NO release (mV/s)
100 $\mu$ M Oxypurinol	0.00
100 $\mu$ M DPI	0.00

There was no release of NO in the presence of the XOR  
5 inhibitors.

Test 5

Full fat milk was assayed with 20mM nitrite and 1mM NADH  
10 as described above in Test 1. The milk had a protein  
content of 3.4g/100ml. 600 $\mu$ l of milk was used in each  
test giving a protein content in each assay of 20.4mg.

The milk was taken and divided into two halves. One  
was kept at 4°C, and the other at -20°C. Samples of the  
15 milk were taken over several days and the generation of  
NO was investigated using the method described above.  
The results are given below in Table 5.

Table 5

20

Day	4°C - NO release (mV/s)	-20°C - NO release (mV/s)
1	77.57	73.00
2	75.50	90.05
3	68.67	52.65
6	626.1	73.35
8	Over 1000	39.20

It was found that the milk which was kept at 4°C  
started to go off after 3 days and that that was



accompanied by a large increase in NO release which was not inhibited by 100 $\mu$ M oxypurinol or DPI. Therefore, when the milk goes off it is probable that bacteria in the milk are starting to produce NO from the nitrite.

5 The milk kept frozen retained its activity and did not go off.

Samples of fresh full fat milk assayed with 100 $\mu$ M Oxypurinol or DPI were tested and found to give no detectable NO generation. Thus full fat milk does include a source of active XOR which produces NO under hypoxic conditions with nitrite and NADH.

10

#### Test 6

Human breast milk was assayed with 20mM nitrite and 1mM NADH using the method as described above. The human breast milk was collected on several days post partum and frozen at -20°C. 600 $\mu$ l of milk was used in each test.

15

20 The generation of NO was investigated using the method described above. The results are given below in Table 6.

Table 6

Days post partum	XOR protein content ( $\mu$ g/ml)	NO release (mV/s)
7	411.26	40.45
30	683.05	60.75
36	561.49	42.48
66	305.52	27.80
158	110.84	29.80

Test 7

A selection of formula feeds (formula milks) were taken and assayed with 20mM nitrite and 1mM NADH as described  
5 above. The formula milks were tested for the generation of NO. The Cow and Gate Premium was left for 7 days after opening and then tested again. The results are shown in Table 7.

10 Table 7

Formula feed	NO release (mV/s)
Cow and Gate Premium (fresh)	0.00
Cow and Gate Premium (7 days old)	Over 1000
Milupa Aptimil First with Milupan	0.00
Sma Wysoy	0.00
Sma Gold	0.00
Farleys First	0.00
Milupa Preaptimil with Milupan	0.00

There was no activity found in any of the fresh formula  
milks. The Cow and Gate formula had gone off after 7  
15 days and the NO release is probably related to bacteria  
(the activity was not inhibited by 100µM oxypurinol or  
DPI).

Test 8

20

To obtain cells used in this test, the infective  
bacterial strain Escherichia coli NCTC 86 (E.coli) was

cultured on nutrient agar at 37° until colony formation occurred, usually overnight. This stock culture was used for subsequent experiments including sub-culturing in nutrient broth and plated onto agar weekly. Experimental  
5 cultures of E.coli were set up overnight in nutrient broth from single colonies on an agar plate. Cells were harvested and counted using a standard curve of known absorbance at 470nm against viable cell count.

10 Peroxynitrite was generated by the method of Crow et al, Biochem. 34, p.3544-3552 (1995). In accordance with that method, a mixture of sodium nitrite and hydrogen peroxide was reacted under acid conditions then immediately  
15 quenched by the addition of sodium hydroxide. The concentration of ONOO<sup>-</sup> formed was measured using an absorption coefficient of 1670 M<sup>-1</sup> cm<sup>-1</sup> in a spectrophotometer at a wavelength of 303nm. Solutions of different concentrations for use in (a) below were obtained by dilution of the product solution using PBS.

20

#### 8(a) Peroxynitrite effect on cell viability

Cells cultured as described above were diluted to suitable working concentrations (10<sup>4</sup> - 10<sup>5</sup> Cells Ml<sup>-1</sup>) in sterile phosphate buffered saline (PBS). ONOO<sup>-</sup> at a range  
25 of concentrations from 100µm to 0.01µm was added as a bolus dose to cells and incubated at room temperature for ten minutes. Aliquots were taken from the cell cultures and plated on to nutrient agar and incubated in a warm room at 37°C overnight. Viable cells formed colonies on  
30 the agar and were counted. The number of colonies formed was related to an untreated control and a graph of the results is shown in Figure 1, which illustrates the effect of ONOO<sup>-</sup> on cell viability. The decrease in viable

count with increasing concentration of  $\text{ONOO}^-$  in Fig. 1 is indicative of a profound inhibitory effect.

#### 8(b) Effect of peroxynitrite addition to bovine milk

5 ~~Commercially produced bovine milk~~ was purchased in semi-skimmed form. Aliquots were taken on day one of experimentation and plated onto nutrient agar and grown overnight at  $37^\circ\text{C}$ . The number of colonies formed following incubation was counted. The remaining milk was  
10 divided into three portions after removal of those aliquots. No peroxynitrite was added to the first portion, which was the control. A single bolus dose of  $100\mu\text{M}$   $\text{ONOO}^-$  was added to the second portion on day one, and the third portion was treated by addition of a daily  
15 bolus dose of  $100\mu\text{M}$   $\text{ONOO}^-$ . The portions were stored during the four days of the test at a temperature of  $4^\circ\text{C}$ . Each day, aliquots were taken from each portion and cultured overnight at  $37^\circ\text{C}$  for viable count determination.

20

The results are shown in Fig. 2 and demonstrate that  $\text{ONOO}^-$  addition to milk caused a reduction in the number of colony forming units (CFU) over time compared to the control. The single bolus addition reduced the CFU  
25 significantly ( $p \leq 0.01$ ) and the multiple dose  $\text{ONOO}^-$  reduced the CFU significantly below control ( $p \leq 0.01$ ) and also significantly below the single dose  $\text{ONOO}^-$  ( $p \leq 0.05$ ).

#### 30 8(c) Effect of xanthine oxidase derived species on cell viability

*E. coli* were cultured as described above. Aliquots were taken and incubated with a reaction system consisting of bovine Xanthine oxidase (XO) ( $53.2 \mu\text{gml}^{-1}$ ), nicotinamide

adenine dinucleotide in reduced form (NADH) ( $300\mu\text{M}$ ), sodium nitrite, ( $\text{NaNO}_2$ )  $1\text{mM}$  and oxygen at a range of concentrations. Desired oxygen concentrations were generated by delivery into the system of a mixture of oxygen and nitrogen in appropriate proportions and determined using a Clark-type  $\text{O}_2$  electrode. This reaction was followed at  $37^\circ\text{C}$  for 30 min with mixing before an aliquot was taken and plated onto agar and incubated in a warm room at  $37^\circ\text{C}$ . Viable cells formed colonies on the agar and were counted. Viable cell counts were performed in triplicate and the results expressed as a percentage viable count in each case related to a non enzyme control of the same oxygen concentration. The results are shown in Fig. 3, which suggest that XO-mediated killing of cells is occurring. The most effective killing (indicated by the lowest viable cell count) is at an oxygen concentration of approximately 3% of saturation. The range of oxygen tensions used covers those in which XO has previously been considered to be active, namely superoxide generation (21%  $\text{O}_2$  saturation) and nitric oxide production (0%  $\text{O}_2$  saturation). A certain amount of killing is seen at both of these extremes as compared with control samples. However, it is only at an intermediate oxygen concentration that the greatest amount of killing is observed. This suggests a role for peroxynitrite mediated killing which has been generated in this system by the enzyme xanthine oxidase.

The results of parts (a) and (b) of this test appear to confirm the bactericidal potency of the peroxynitrite species. Part (c) above supports the hypothesis that, under appropriate conditions, XOR can catalyse production both of superoxide and of  $\text{NO}$ , interaction of those two

products giving rise to peroxynitrite, and possibly other interaction products that may have similar bactericidal activity to peroxynitrite. The oxygen concentration of 3% at which maximum killing was observed is believed to be similar to that in the neonatal gut.

#### Example 1

The effect of the addition of XOR to formula feed on the generation of NO was investigated. Different compositions comprising one or more of buttermilk, formula feed, XOR, nitrite and NADH were studied under hypoxic conditions.

The samples were tested for NO generation using the method described above.

To the "milk" bijou were added either 598µl Cow and Gate formula feed and 2µl XOR or 100µl buttermilk and 500µl 1X PBS to give a total volume in the "milk" bijou of 600µl in each case.

Table 8

Sample	20mM nitrite added?	1mM NADH added?	NO release (mV/s)
formula feed and XOR	Yes	Yes	247.3
formula feed and XOR	No	No	0.0
formula feed and XOR	Yes	No	0.0
formula feed and XOR	No	Yes	0.0
formula feed and 1X PBS	Yes	Yes	0.0
Buttermilk	Yes	Yes	379.1

Table 8 shows that where no XOR is added to the  
5 formula feed, there is no generation of NO. That is  
consistent with the applicant's findings that formula  
feeds do not contain active XOR.

The addition of XOR to the formula feed in the  
presence of nitrite and NADH led to the generation of NO.  
10 The omission of either the nitrite or the NADH gave no NO  
generation. As indicated above, it is believed that both  
a source of nitrite and an electron donor substrate will  
be available *in vivo* in the GI tract and that in many  
cases specific further addition of nitrite and/or NADH  
15 (or other substrate) will not be required.

Buttermilk alone, without the addition of any of  
XOR, nitrite or NADH, led to the generation of NO. As  
indicated above, buttermilk contains active XOR as well  
as a source of nitrite, and an electron donor substrate.

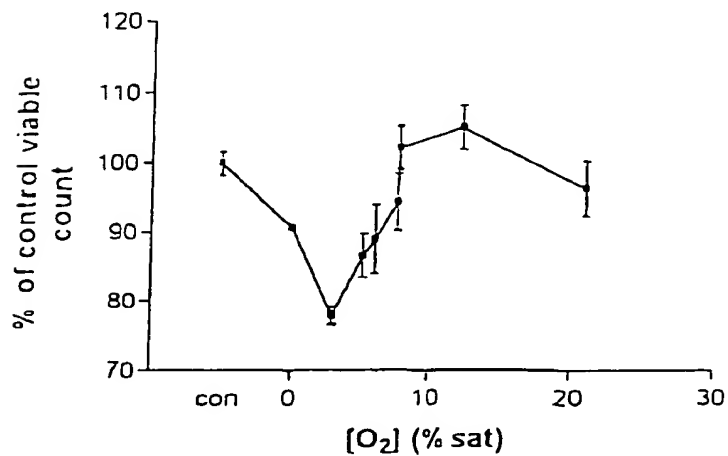
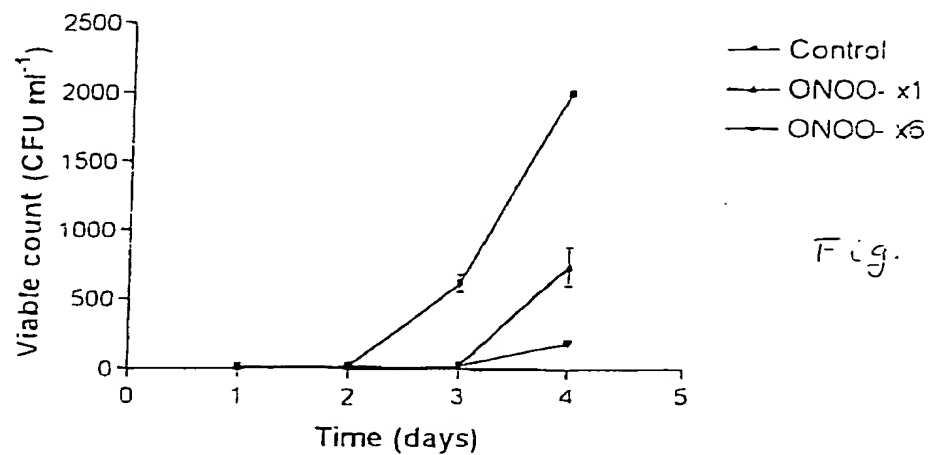
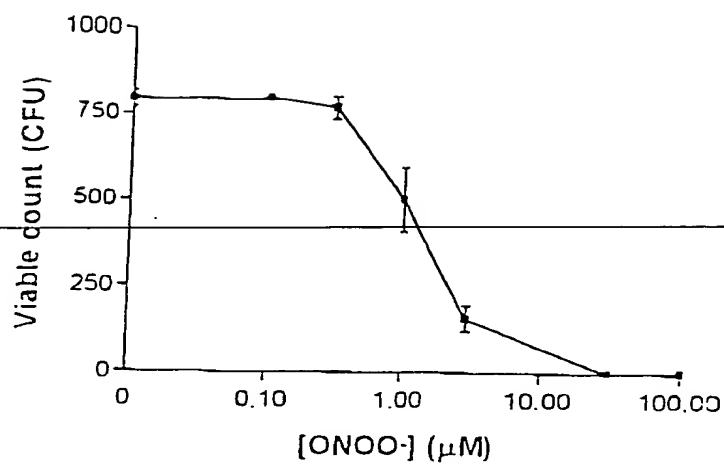
Claims:

1. A formulation for use as a feed, the formulation including active xanthine oxidoreductase (XOR).
- 5 2. A formulation according to claim 1, in which the formulation is for use as formula feed.
3. A formulation according to claim 1 or claim 2, in which the formulation includes from 50 to 150 $\mu$ g/ml of XOR.
- 10 4. A formulation according to any one of claims 1 to 3, in which the formulation includes buttermilk, the buttermilk including active XOR.
5. A formulation according to any one of claims 1 to 4, in which the formulation is in liquid form.
- 15 6. A formulation according to any one of claims 1 to 5, in which the formulation comprises two separate portions, the first portion including active XOR and the second portion comprising substantially no active XOR.
7. A formulation according to claim 6, in which the  
20 second portion is in the form of a powder.
8. A formulation according to claim 6 or claim 7, in which the second portion has been heat treated.
9. A formulation according to any one of claims 6 to 8, in which the first portion has been pasteurised.
- 25 10. A formulation according to any one of claims 6 to 9, in which the first portion is in a first container, the second portion is in a second container.
11. A formulation according to any one of claims 1 to 10, the formulation further including one or more  
30 electron donors.
12. A formulation according to any one of claims 1 to 11, the formulation further including one or more electron acceptors.



13. A composition for addition to a formulation for use as feed, the composition comprising active XOR.
14. A composition according to claim 13, the composition comprising buttermilk.
- 
- 5 15. A composition according to claim 13 or claim 14, the composition being in the form of a powder.
16. A composition according to any one of claims 13 to 15, in which the composition further includes one or more electron donors.
- 10 17. A composition according to any one of claims 13 to 16, in which the composition further includes one or more electron acceptors.
18. Method of making a formulation for use as feed, the method comprising the step of adding a composition
- 15 comprising active XOR.
19. A method according to claim 18, the composition being according to any one of claims 13 to 17.
20. A method according to claim 18 or claim 19, the method comprising the steps of:
- 20     a. preparing a first portion of the formulation, the first portion comprising a composition including active XOR; and
- b. preparing a second portion of the formulation, the first portion and second portion being separate from
- 25 each other for subsequent mixing to form the formulation.
21. A method according to claim 20, in which the first portion comprises lyophilised buttermilk.
22. A method according to claim 20 or claim 21, in which the second portion comprises a treated feed composition.
- 30 23. Use of active XOR in the treatment of gastrointestinal (GI) infection.
24. Use of active XOR in the treatment of Scours disease.

25. Use of active XOR in the killing of bacteria.
  26. Method of feeding a patient with enteral feed, in which the enteral feed includes active XOR.
  27. Method of feeding a neonate with formula feed, in
- 
- 5 ~~which the formula feed includes active XOR.~~



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Abel + Murray

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